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SUBSTITUTE FORM PTO-1390 U S DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER
11283-020US1

U.S. APPLICATION NO. (If Known, see 37 CFR 1.5)
10/089212

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/JP00/01969	29 March 2000	October 8, 1999

TITLE OF INVENTION
MODULATE APTAMER AND METHOD OF DETECTING TARGET PROTEIN BY USING THE SAME

APPLICANT(S) FOR DO/EO/US
Penmetcha Kumar and Rika Yamamoto

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☐ This is an express request to promptly begin national examination procedures (35 U.S.C. 371(f)).
4. ☐ The US has been elected by the expiration of 19 months from the priority date (PCT Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. ☐ is attached hereto (required only if not communicated by the International Bureau).
   b. ☒ has been communicated by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. ☒ are attached hereto (required only if not communicated by the International Bureau).
   b. ☐ have been communicated by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☐ have not been made and will not be made.
8. ☐ An English language translation of amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☐ An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11 to 16 below concern other documents or information included:

11. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☒ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☒ A FIRST preliminary amendment.
    ☐ A SECOND or SUBSEQUENT preliminary amendment.
14. ☐ A substitute specification.
15. ☐ A change of power of attorney and/or address letter.
16. ☐ Other items or information:
    - ☒ International Search Report
    - ☒ WO 01/27263
    - ☒ PH-933-PCT
    - ☒ Form PCT/IPEA/416
    - ☒ PCT/IB/308 and PCT/IPEA/409

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March 26-02 — Date of Deposit
Francine Rose — Signature
Francine Rose — Typed Name of Person Signing

U.S. APPLICATION NO. (IF KNOWN) 10/089212	INTERNATIONAL APPLICATION NO. PCT/JP00/01969	ATTORNEY'S DOCKET NUMBER 11283-020US1

17. ☐ The following fees are submitted:

Basic National Fee (37 CFR 1.492(a)(1)- (5)):

Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO **$1040**

International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO **$890**

International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO..... **$740**

International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4)..... **$710**

International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4)..... **$100**

	CALCULATIONS PTO USE ONLY	
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Surcharge of **$130** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$0.00	

Claims	Number Filed	Number Extra	Rate		
Total Claims	23 - 20 =	3	x **$18**	$54.00	
Independent Claims	1 - 3 =	0	x **$84**	$0.00	
MULTIPLE DEPENDENT CLAIMS(S) (if applicable)			+ **$280**	$280.00	
			TOTAL OF ABOVE CALCULATIONS =	$1,224.00	
☐ Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2.				$0.00	
			SUBTOTAL =	$1,224.00	
Processing fee of **$130** for furnishing the English Translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(f))				$0.00	
			TOTAL NATIONAL FEE =	$0.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property +				$40.00	
			TOTAL FEES ENCLOSED =	$1,264.00	
			Amount to be refunded:		$
			Charged:		$

a. ☒ A check in the amount of $1,264.00 to cover the above fees is enclosed.
b. ☐ Please charge my Deposit Account No. 06-1050 in the amount of $0.00 to cover the above fees. A duplicate copy of this sheet is enclosed.
c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 06-1050. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

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SIGNATURE :

Chris T. Mizumoto
NAME

42,899
REGISTRATION NUMBER

Attorney's Docket No.: 11283-020US1 / PH-933PCT-US

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	: Kumar et al.	Art Unit	: Unknown
Serial No.	: To be determined	Examiner	: Unknown
Filed	: March 26, 2002		
Title	: MODULATE APTAMER AND METHOD OF DETECTING TARGET PROTEIN BY USING THE SAME		

Commissioner for Patents
Washington, D.C. 20231

PRELIMINARY AMENDMENT

Prior to examination, please amend the application as follows:

In the claims:

Amend claim 5, 12, and 14 as follows:

-- 5. (Amended) The modulate aptamer according to claim 1, wherein the target protein is HIV-1 Tat protein and/or a fragment thereof.

12. (Amended) The method according to claim 10, wherein the immobilization is by specific binding between avidin or streptoavidin and biotin.

14. (Amended) The method according to claim 9 or 10, wherein the target protein is an HIV-1 Tat protein and/or a fragment thereof. --

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March 26, 2002
Date of Deposit

Signature

Francisco Robles
Typed or Printed Name of Person Signing Certificate

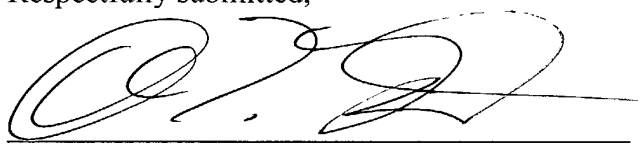
REMARKS

Claims 5, 12, and 14 have been amended to eliminate multiple-on-multiple dependencies.

Attached is a marked-up version of the changes being made by the current amendment.

Applicant asks that all claims be examined. If necessary, please apply charges or credits to Deposit Account No. 06-1050.

Respectfully submitted,



Date: March 26, 2002

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New York, New York 10111
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Version with markings to show changes made

In the claims:

Claims 5, 12, and 14 have been amended as follows:

5. (Amended) The modulate aptamer according to claim 1 [any one of claims 1 to 4], wherein the target protein is HIV-1 Tat protein and/or a fragment thereof.

12. (Amended) The method according to claim 10 [or 11], wherein the immobilization is by specific binding between avidin or streptoavidin and biotin.

14. (Amended) The method according to [any one of claims 9 to 13] claim 9 or 10, wherein the target protein is an HIV-1 Tat protein and/or a fragment thereof.

12/pr

DESCRIPTION

MODULATE APTAMER AND METHOD OF DETECTING TARGET PROTEIN BY USING THE SAME

TECHNICAL FIELD

The present invention relates to a modulate aptamer which binds specifically to a specific target protein, in particular, Tat protein of human immunodeficiency virus type-1 (HIV-1), and a method and a kit for detecting a target protein, in particular HIV-1 Tat protein, using the same.

BACKGROUND ART

It has been reported that among nucleic acid ligands (aptamers), there are those having affinity/specificity for a protein comparable to the affinity/specificity of an antibody for its antigen, and it is expected that these can be used as a molecule recognition factor in a biosensor. (Osborn, S.E. and Ellington, A.D., *Chem. Rev.* 97, 349-370 (1997)). To this end, research conducted using full-length aptamer has been promoted and carried out (Drolet, D.W., Moon-McDermott, L. and Roming, T.S. *Nature Biotechnol.* 14, 1021-1025 (1996); Kleinjung, F. et al, *Anal. Chem.* 70, 328-331 (1998); Potyrailo, R.A., Conrad, C.R., Ellington, A.D. and Hieftje, G.M. *Anal. Chem.* 70, 3419-3425 (1998)).

Further, it was thought that an aptamer exhibiting high affinity with Tat protein of HIV-1 and HIV-2 has sensitivity sufficient to measure the amount of Tat protein released from infected cells or in the sera of infected patients. (Westendrop, M.O. et al, *Nature* **375**, 497-500 (1995); Pantaleo, G. et al, *Nature* 362, 355-358 (1993); Embretson, J. et al, *Nature* 362, 359-362 (1993)).

Recently, a nucleic acid motif having a stem-loop structure called a molecular beacon was developed as a tool for finding complementary target sequences (Tyagi, S. and Kramer, F.R. (1996) Nature Biotechnology 14, 303-308). This molecular

beacon includes two structural components: a loop sequence being a probe complementary to a target sequence, and a stem structure formed by annealing of complementary arm sequences on the both ends of the probe sequence. A fluorescent probe is attached to the end of one arm forming the stem structure by a covalent bond, and a quencher substance is attached to the end of the other arm by a covalent bond. The fluorescent probe and quencher substance are in close proximity due to the stem formation of this beacon, and as a result, fluorescence is not emitted. When this molecular beacon encounters a target molecule, it forms a probe (loop structure)-target hybrid that is more stable than the stem structure of the molecular beacon. As a result of a structural change in the stem-loop motif, the two arm sequences move away from one another, enabling fluorescence to be emitted. Therefore, using a molecular beacon, it is possible to detect a specific nucleic acid in real-time without stopping the reaction. Further, it is reported that this can be applied to live cells. (Tyagi, S. and Kramer, F.R. (1998) Nature Biotechnology 16, 49-53; Matsuo, T. (1998) Biochem. Biophys Acta. 1379, 178-184; Sokol, D.L., Zhang, X., Lu, P. and Gewirtz, A.M. (1998) Proc Natl. Acad. Sci. USA 95, 11538-11543).

However, there were a number of limitations to this first approach described above. Examples of such limitations include the fact that the longer the length of the aptamer, the lower the efficiency of chemical synthesis; the necessity of protecting the full-length aptamer against nuclease; and the fact that in the case of a plurality of analyses, with long aptamers the structuring/restructuring efficiency is low. That is, for reasons of the size and recognition mode of aptamers, there was a limit to the application of aptamers to experiments using biosensors.

DISCLOSURE OF THE INVENTION

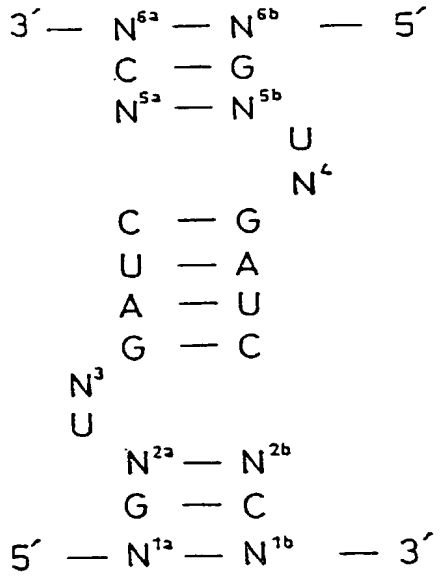
The present inventors solved the above problem, and developed a novel strategy which enables use as an effective biosensor by using an aptamer which forms a conjugate and stabilizes only in the presence of a target protein or substance to be analyzed. Specifically, based on an aptamer RNA for an HIV-1 Tat protein which was

the subject of an earlier patent application (Japanese Patent Laid-Open Application No. Hei 11-127864), the aptamer sequence was divided onto independent double strands and a modulate aptamer more effective in detection of Tat protein was constructed.

It was found that the HIV-1 Tat aptamer RNA-derived modulate aptamer according to the present invention forms a conjugate in the presence of HIV-1 Tat protein or Tat-derived peptide at 1 nM or less, whereas it does not form a conjugate in the presence of other RNA-binding proteins or nuclear extracts.

That is, the present invention relates to a modulate aptamer being an aptamer constituted by two complementary oligonucleotide chains which forms a conjugate and stabilizes only in the presence of a target protein, and particularly relates to the modulate aptamer for which the target protein is HIV-1 Tat protein and/or a fragment thereof.

As an example of the modulate aptamer of the present invention, a modulate aptamer having the nucleotide sequence represented by the following secondary structure (I) is provided.

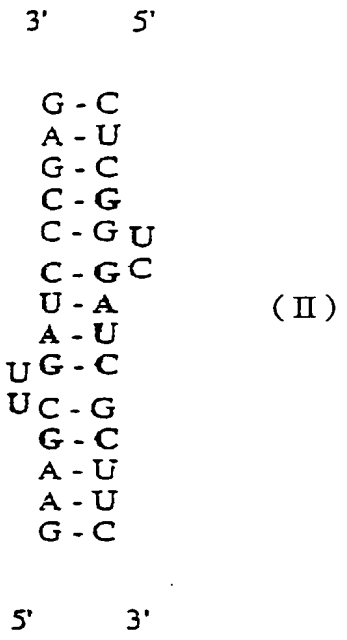


(I)

(In the structure, N^{1a} and N^{1b} represent at least 1 pair of nucleobases capable of complementary base pair formation; N^{2a} and N^{2b} represent at least 1 pair of nucleobases capable of complementary base pair formation; N^3 and N^4 each independently represent

1 or 2 nucleobases; N^{5a} and N^{5b} represent at least 1 pair of nucleobases capable of complementary base pair formation; N^{6a} and N^{6b} represent at least 1 pair of nucleobases capable of complementary base pair formation; and solid lines represent hydrogen bonds between nucleobases.)

As a particularly preferable modulate aptamer according to the present invention, a modulate aptamer having the nucleotide sequence represented by the following secondary structure (II) is provided.



(II)

(In the structure, solid lines represent a hydrogen bond between nucleobases.)

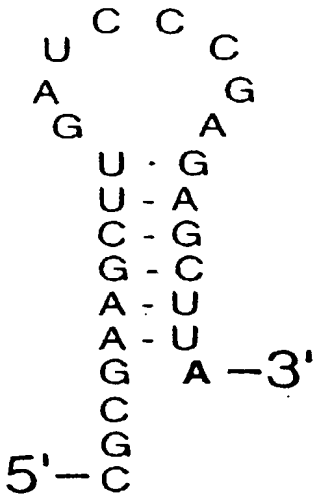
Further, using the above-mentioned modulate aptamer, a method enabling detection of a target protein such as an HIV-1 Tat protein, simply and with high sensitivity, specifically, a micro-titer plate assay using fluorescence, was developed. It is thought that there is the possibility that this assay can be used in a manner similar to a DNA array, and can be applied and extended to detection of virus proteins and other small molecule substances.

Further, as one embodiment of the present invention, there is provided a modulate aptamer in which one of the oligonucleotide chains constituting the modulate

aptamer has, intramolecularly, mutually complimentary sequences of four or more consecutive nucleotides, and which adopts a stem-loop structure in the absence of a target protein.

Specifically, for example, when a fluorescent substance is bound to the 5' or 3'-end of the oligonucleotide chain of the stem-loop structure, and a quencher substance in respect of the fluorescent substance is attached to the 3'or 5'-end, respectively, such that it forms a conjugate only in the presence of a target protein and emits fluorescence, then detection becomes very easy.

As a preferable oligonucleotide chain of this stem-loop structure, one which has the nucleotide sequence represented by the following secondary structure (III) is provided.



(III)

(In the structure, solid lines represent hydrogen bonds between nucleobases.)

This novel strategy for "modulate aptamers" can be easily applied to a biosensor for characterizing molecule recognition factors for various molecules.

Therefore, the present invention further provides a method of detecting a target protein, particularly an HIV-1 Tat protein and/or fragment thereof, which comprises radioactively or non-radioactively labeling one oligonucleotide chain of the modulate aptamer, and detecting the presence and/or amount of the target protein with the presence or absence, and/or amount of a conjugate formed in the presence of the

target protein.

In one embodiment of the method of the present invention, the non-radioactive label is fluorescein, and the conjugate is detected by a fluorescent signal thereof.

Further, the present invention provides a method of detecting a target protein, particularly, an HIV-1 Tat protein and/or fragment thereof, which comprises immobilizing one oligonucleotide chain of the modulate aptamer on a support, and detecting the presence and/or amount of the target protein with the presence or absence, and/or amount of a conjugate formed by addition of the other oligonucleotide chain labeled radioactively or non-radioactively.

In one embodiment of the method of the present invention, the immobilization is by specific binding between avidin or streptoavidin and biotin.

According to the present invention, there is further provided a kit for detecting a target protein, particularly, an HIV-1 Tat protein and/or fragment thereof, wherein the kit comprises a support, one oligonucleotide chain of the modulate aptamer to be immobilized on the support, and the other oligonucleotide chain which forms a conjugate in the presence of the target protein.

Specifically, in the kit of the present invention, the one oligonucleotide chain is the 5'-chain (left side chain in Fig. 2) or 3'-chain (right side chain in Fig. 2) of the modulate aptamer, and the other oligonucleotide is the 3'-chain or 5'-chain, respectively.

The contents described in the specification and/or drawings of Japanese Patent Application No. Hei 11-288677, which forms the basis of the priority right of the present application, are incorporated herein in their entirety.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the secondary structures of aptamer RNA^{Tat}, TAR-1RNA (59mer) and TAR-2 RNA (123mer). The portion framed in solid lines shows the core factor necessary for Tat binding.

Figure 2 shows the secondary structures of the modulate aptamer RNAs of the present invention (i, DA-1/DA-2; ii, DA-3/DA-4; iii, DA-5/DA-6; iv, DA-7/DA-8; v, DA-1/DA-4).

Figure 3 shows an autoradiogram of various modulate aptamer RNAs. Lane 1: radioactively labeled 5'-oligo (10 nM) only; lane 2: radioactively labeled 5'-oligo (10 nM) and unlabeled 3'-oligo (200 nM); lane 3: radioactively labeled 5'-oligo (10 nM) and unlabeled 3'-oligo (200 nM) in the presence of 20 nM CQ; lane 4: radioactively labeled 5'-oligo (10 nM) and unlabeled 3'-oligo (200 nM) in the presence of 200 nM Tat-1. A thick arrow shows the position of a modulate aptamer RNA-Tat or modulate aptamer RNA-CQ conjugate. The position of free 5'-oligonucleotide is shown with a thin arrow.

Figure 4 shows an autoradiogram obtained by a binding analysis using gel shift assay of modulate aptamer DA-1/DA-2 and CQ, and a saturation curve and Scatchard plot of modulate aptamer DA-1/DA-2-CQ. Thick arrow and thin a rrow show the positions of modulate aptamer RNA-CQ conjugate and free 5'-olig onucleotide, respectively.

Figure 5 shows an autoradiogram obtained by a binding analysis using gel shift assay of modulate aptamer DA-5/DA-6 and CQ, and a saturation curve and Scatchard plot of modulate aptamer DA-5/DA-6-CQ. Thick arrow and thin a rrow show the positions of modulate aptamer RNA-CQ conjugate and free 5'-olig onucleotide, respectively.

Figure 6 shows the secondary structure of an inactive-form modulate aptamer (DA-5i/DA-6i) (A) and an autoradiogram obtained by analysis of the binding of the aptamer with CQ peptide using gel shift assay (B). Lane 1: radioactively labeled 5'-oligo (10 nM); lane 2: radioactively labeled 5'-oligo (10 nM) and unlabeled 3'-oligo (200 nM); lane 3: radioactively labeled 5'-oligo (10 nM) and unlabeled 3'-oligo (200 nM) in the presence of 20 nM CQ.

Figure 7 shows the secondary structure of double stranded TAR RNA (ii,DT-1/DT-2) (A) and an autoradiogram obtained by analysis of the binding of t

he RNA with CQ peptide using gel shift assay (B). Lane 1: radioactively labeled 5'-oligo (10 nM); lane 2: radioactively labeled 5'-oligo (10 nM) and unlabeled 3'-oligo (200 nM); lane 3: radioactively labeled 5'-oligo (10 nM) and unlabeled 3'-oligo (200 nM) in the presence of 20 nM CQ.

Figure 8 shows an autoradiogram obtained by a gel shift assay of t he binding of modulate aptamer DA-5/DA-6 and Tat-derived peptides (CQ, RE, C P). Thick arrow and thin arrow show the positions of modulate aptamer RNA-CQ conjugate and free 5'-oligonucleotide, respectively.

Figure 9 shows an analyte-dependent hybridizing oligonucleotide assay (ADHONA). This figure shows the secondary structure of modulate aptamer DA-9/DA-10 for use in ADHONA (A) and the mechanism of ADHONA (B)

Figure 10 shows graphs indicating CQ peptide concentration dependency of ADHONA: fluorescent signal intensity in the absence of CQ peptide (control) or in the presence of CQ peptide (10, 50, 100 pmol). Results are indicated as an average (± standard deviation) of 3 experiments.

Figure 11 shows the results of ADHONA using Tat-1 peptide: fluorescent signal intensity in the absence of Tat-1 or CQ peptide (control); fluorescent signal intensity in the presence of 10pmol CQ peptide (1) ; fluorescent signal intensity in the presence of 200pmol Tat-1 protein (2). Results are indicated as an average (±standard deviation) of 3 experiments.

Figure 12 shows the influence on ADHONA by co-existence of HeLa nuclear extract: fluorescent signal intensity in the absence of Tat-1 or CQ peptide (control); fluorescent signal intensity in the presence of 8 units of HeLa nuclear extract (1); fluorescent signal intensity in the presence of 10pmol CQ peptide (2). Results are indicated as an average (±standard deviation) of 3 experiments.

Figure 13 shows the secondary structure of oligonucleotide chain DA13(C-A) of a stem-loop structure having fluorescein bound to the 5'-end and DABCYL bound to the 3'-end, the secondary structure of the DA13(C-A)/DA6 which is a modulate aptamer RNA of the present invention, and nucleotide pair formation

between DA13(C-A) and completely complementary DA13C.

Figure 14 shows detection by fluorescence of CQ using a molecular beacon aptamer, and a model of the secondary structure of the oligonucleotide chain thereupon. 10nM DA13(C-A) only (Control); 10nM DA13(C-A)+100nM DA6(CQ-); 10nM DA13(C-A)+100nM DA6+100nM CQ peptide (CQ+); 10nM DA13(C-A)+100nM DA13C(DA13C).

Figure 15, shows the result of measuring fluorescence intensity of samples CQ-, CQ+ and DA13C under the same conditions as for Fig. 14 using FluorImager (Molecular Dynamics). Results indicate the average (±standard deviation) of three experiments.

Explanation of sequence listing

SEQ ID NO:1: aptamer RNA for an HIV-1 Tat protein

SEQ ID NO:7: 5'- side oligonucleotide of a modulate aptamer

SEQ ID NO:8: 5'-side oligonucleotide of a modulate aptamer

SEQ ID NO:9: 5'- side oligonucleotide of a modulate aptamer

SEQ ID NO:10: 5'- side oligonucleotide of a modulate aptamer

SEQ ID NO:11: 3'-side oligonucleotide of a modulate aptamer

SEQ ID NO:12: 3'-side oligonucleotide of a modulate aptamer

SEQ ID NO:13: 3'-side oligonucleotide of a modulate aptamer

SEQ ID NO:14: 3'-side oligonucleotide of a modulate aptamer

SEQ ID NO:15: modified 5'-side nucleotide

SEQ ID NO:16: modified 3'-side nucleotide

SEQ ID NO:17: modified 5'-side nucleotide

SEQ ID NO:18: 5'- side oligonucleotide of a modulate aptamer

SEQ ID NO:19: 3'-side oligonucleotide of a modulate aptamer

SEQ ID NO:20: molecular beacon aptamer

SEQ ID NO:21: complementary oligonucleotide to a molecular beacon aptamer

BEST MODE FOR CARRYING OUT THE INVNETION

Below, the present invention will be explained in detail.

In the present invention, an "aptamer" refers to a nucleic acid ligand artificially engineered to bind strongly and specifically with a particular target protein. A "modulate aptamer" refers to an aptamer wherein the core portion of the aptamer is designed such that it has a low Tm value and divided in two short oligonucleotide chains which bind to form a double strand only in the presence of a target protein. A "molecular beacon aptamer" refers to an aptamer which comprises two structural components, a loop sequence which becomes a complementary probe to a target sequence, and a stem structure which is formed by annealing of two complementary arm sequences present on both ends of the probe sequence.

Further, in the present invention, "complementary" and "complementary base pair formation" refer to pairing between nucleobases: adenine and uracil, and guanine and cytosine by a hydrogen bond. Examples of nucleobases capable of complementary base pair formation include the combinations of adenine and uracil, and guanine and cytosine.

A "conjugate" refers to a formation in a stabilized state between a double stranded modulate aptamer according to the present invention and a target protein via hydrogen bonds and/or non-covalent bonds such as hydrophobic interactions. Here, "stabilized" refers to formation of a conjugate in which the binding-dissociation equilibrium is biased toward a bound state, and dissociation is difficult.

"Radioactive labeling" or "Radioactively label" refers to labeling using a substance including radioactive isotopes such as ^{3}H, ^{13}C, ^{32}P, etc., and "non-radioactive labeling" or "non-radioactively label" refers to labeling not using radioactive substances. In non-radioactive labeling, labeling substances specifically include, but are not particular limited to luminescent molecules, fluorescent molecules such as fluorescein, enzymes such as peroxidase and alkali phosphatase, antibodies, and other molecules having binding specificity to specific molecules such as a biotin that are used in the art.

A "support" refers to objects used in the detection method and detection kit

according to the present invention to perform conjugate formation reaction at an immobile position, and may be anything which does not exhibit absorbance such as glass or plastic. In the assay of the present invention using a minute amount of reaction solution, a microtiter plate and the like is particularly preferable.

A "target protein" refers to a protein or peptide which has high affinity and specificity for the modulate aptamer of the present invention, binds therewith and stabilizes, and the presence or absence, and/or amount thereof can be detected thereby. Examples include HIV-1 Tat and a fragment thereof having an amino acid chain length of approximately 40, CQ peptide, RE peptide, etc., and HIV Rev protein etc. Further, as HIV, HIV-1 and HIV-2 are known. Therefore, as used herein Tat-1 and TAR-1 respectively refer to HIV-1 Tat (trans activating factor) protein and TAR (trans activation response region) and Tat-2 and TAR-2 respectively refer to HIV-2 Tat protein and TAR.

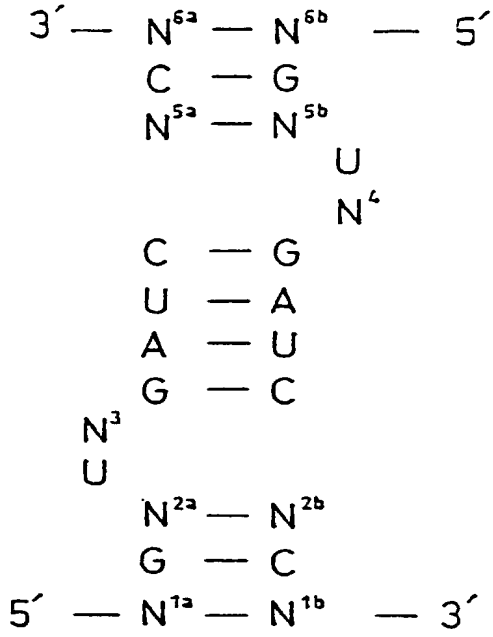
The present inventors have previously isolated aptamer RNA^{Tat} (Fig. 1 left, SEQ ID NO:1) which binds to HIV-1 Tat protein with higher specificity and affinity than HIV-1 TAR (Fig. 1 center), and reported the use thereof in a pharmaceutical composition for diagnosis, prevention and treatment of deseases in which HIV is involved (Yamamoto, R., Murakami, K., Taira, K. and Kumar, P.K.R., *Gene Ther. Mol. Biol.* **1**, 451-466 (1998), Patent Application Laid-Open No.: Hei 11-127864). This aptamer has high affinity for Tat protein (relative to Tat-1, Kd value is 100 times lower than TAR-1 RNA (59 mer, Fig. 1 center, SEQ ID NO:2), and relative to Tat-2, Kd value is 40 times lower than TAR-2 RNA (123 mer, Fig. 1 right, SEQ ID NO:3)), and since the sequence of the loop portion of the aptamer sequence is not the Tat protein binding site, it is possible to divide the aptamer into two strands.

From this viewpoint, the present inventors synthesized several modulate aptamer oligonucleotides having varying lengths and comprising core binding factor (the portion framed by solid lines in Fig. 1) relative to Tat-1 protein.

That is, first, the oligonucleotide chain corresponding to the 5'-side of the modulate aptamer of the present invention and the oligonucleotide chain corresponding

to the 3'-side, were respectively chemically synthesized. Upon use, one or both of the two complementary oligonucleotide chains may be detectably labeled radioactively or non-radioactively as defined above and used.

The modulate aptamer of the present invention is reconstituted from the above oligonucleotide chains in the presence of a target protein. A preferable example thereof has the structure represented by the following secondary structure (I).



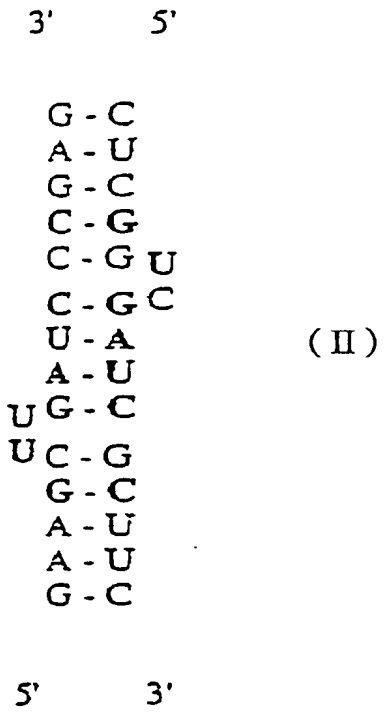
(I)

(In the structure, N^{1a} and N^{1b} represent at least 1 pair of nucleobases capable of complementary base pair formation; N^{2a} and N^{2b} represent at least 1 pair of nucleobases capable of complementary base pair formation; N^3 and N^4 each independently represent 1 or 2 nucleobases; N^{5a} and N^{5b} represent at least 1 pair of nucleobases capable of complementary base pair formation; N^{6a} and N^{6b} represent at least 1 pair of nucleobases capable of complementary base pair formation; and solid lines represent hydrogen bonds between nucleobases.)

The modulate aptamer of the present invention represented by the above secondary structure (I), exhibits high affinity to HIV-1 Tat protein by having the core factor of TAR-1 RNA repeated and arranged in the opposite direction, and binds sequence specifically to Tat-1 protein or Tat-derived peptides such as CQ, etc. Further, it

was apparent that the presence of bulge residues protruding from the complementary base pair formation is essential for recognition of HIV-1 Tat protein and efficient binding.

Further, an example of one particularly preferable modulate aptamer in the present invention, is one having the nucleotide sequence represented by the f ollowing secondary structure (II):



(II)

(In the structure, solid lines represent hydrogen bonds between nucleobases.)

From earlier biochemical experiments, the minimum necessary region in HIV-1 Tat protein for RNA recognition and binding was known. This peptide region called CQ peptide consists of amino acid residues 37-72 (SEQ ID NO:4), and binds to TAR-1 RNA with efficiency identical to that of full-length Tat-1 protein (Weeks, K.M., Ampe, C., Schultz, S.C., Steitz, T.A. and Crothers, D.M., *Science* **249**, 1281-1285 (1990); Calnan, B.J., Biancalnan, S., Hudson, D. and Frankel, A.D., *Genes Dev.* 5, 201-210 (1991)). From this fact, experiments to examine the ability of a modulate aptamer were performed using Tat-1 protein or Tat-derived peptides such as CQ as a

target protein. Further, as other peptides, RE derived from Tat-1 peptide (SEQ ID NO:5), CP derived from Tat-2 peptide (SEQ ID NO:6) were also used.

Further, in the present invention, there is provided a novel method of detecting a target protein using a modulate aptamer RNA which forms a conjugate only in the presence of the target protein. This method was analyzed particularly using HIV-1 Tat protein as a target. The modulate aptamer of the present invention form a conjugate efficiently in the presence of Tat protein, and does not form a conjugate in the presence of other RNA-binding proteins. Therefore, the presence and amount of Tat protein can be measured using the modulate aptamer.

In one embodiment of the present invention, one of the oligonucleotide chains of the modulate aptamer of the present invention is radioactively labeled as defined above, and a conjugate is formed in the presence of the other oligonucleotide chain and the target protein, and the presence or absence and/or amount of the conjugate formation is detected. Conditions for conjugate formation reaction are, for example, in a Tris buffer of pH 7 to 8, at 30℃, for 30 minutes, however, conditions such as reaction temperature, reaction time and the composition of the reaction solution can be modified as appropriate by a person skilled in the art.

After formation of a conjugate, it is possible to directly detect formation of the conjugate, however, in some cases, the formed conjugate may be separated by methods normally used in the art such as by electrophoresis.

A non-radioactive label may be used instead of the radioactive label, and for example where fluorescein is used, it can be detected by its fluorescent signal.

In another embodiment of the method of the present invention, one of the oligonucleotide chains is immobilized on a support as defined above, the conjugate formed by addition of the other radioactively or non-radioactively labeled chain in the presence of a target protein is detected. In this case, after conjugate formation reaction, oligonucleotides not forming conjugates can be removed by washing as required prior to detection. Immobilization may be performed by any method normally used in the art, such as by physical or chemical means, and there is no particular limitation on the

method to be used. However, it is preferable to exploit specific binding between avidin or streptoavidin and biotin.

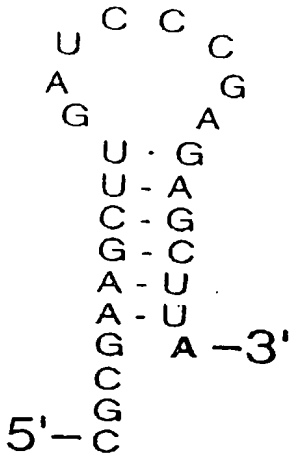
Further, the present invention provides a kit for detecting a target protein, which comprises the support, one oligonucleotide chain of the modulate aptamer to be immobilized on the support, and the other radioactively or non-radioactively labeled oligonucleotide chain which forms a conjugate in the presence of the target protein. Immobilization on the support may be performed at the time of use, however, a pre-immobilized oligonucleotide may be provided in a kit. Similarly to the above, immobilization may be of any type normally used in the art, and is not particularly limited, however, it is preferable to exploit specific binding between avidin or streptoavidin and biotin.

The specificity of the modulate aptamer of the present invention is preserved in a crude sample such as a HeLa nuclear extract, and the detection method of the present invention can be used directly in infected cells and the like.

By the present invention, it was possible to efficiently regulate the formation of a stable double stranded structure of aptamer-derived oligonucleotides in the presence of a target protein. Thereby, the present invention is the first to provide a method of detecting a target protein, particularly Tat protein, or a fragment thereof using a molecular beacon aptamer.

The molecular beacon aptamer used in the present invention, is used in the above described embodiment of a modulate aptamer, and is characterized in that one of the oligonucleotide chains constituting this modulate aptamer has mutually complementary nucleotide sequences consisting of four or more consecutive nucleotides, and has a stem-loop structure in the absence of a target protein. If the mutually complementary sequences consist of three nucleotides of less, a stable stem-loop structure cannot be adopted, which is not preferable.

One example of this stem-loop structure is one having the nucleotide sequence represented by the following secondary structure (III).



(III)

(In the structure, solid lines represent hydrogen bonds between nucleobases.)

In the present invention, when using a molecular beacon aptamer, a fluorescent substance is bound to the 5' or 3'-end of the oligonucleotide chain of the stem-loop structure, and a quencher substance against the fluorescent substance is bound to the 3' or 5' end, respectively.

Specifically, based on the sequence of the modulate aptamer, a quencher substance such as [4'-(4'-dimethyl-aminophenyl-azo) benzoic acid (DABSYL)] is connected by a covalent bond to 5' end of one of the oligonucleotide chains constituting the aptamer, i.e. the sequence which adopts a stem-loop structure, and to the 3'-end, a fluorescent substance such as fluorescein, cumarin, tetramethyl rhodamine etc., thereby constructing a molecular beacon aptamer (Fig. 13 left). As a control, an RNA oligonucleotide having a sequence that is completely complementary to this molecular beacon aptamer was synthesized, which can be used to evaluate the relative amount of fluorescence intensity when the fluorescent substance and the quencher substance are separated by a distance.

DA-13 (C-A) which constitutes one of the oligonucleotides of the modulate aptamer (Fig. 13 left), undergoes a structural change from a stem-loop structure to a double-stranded structure only in the presence of both the probe (the other chain of the modulate aptamer) and the target protein. In the absence of the probe or the target protein, this structural change does not occur. Therefore, in the present invention, the

molecular beacon aptamer undergoes structural change in the presence of a target protein and this change can be detected efficiently.

The above described molecular beacon aptamer is one embodiment of the above described modulate aptamer, and it can be understood that the above described method and kit for detecting a target protein can be appropriately provided by using the molecular beacon aptamer in a similar manner.

Below, the present invention is further explained through the use of Examples, however, the present invention should not be taken to be limited by these Examples.

Example 1:

Synthesis of Tat-derived peptide, aptamer, and modulate aptamer

Chemical synthesis of each of Tat-1-derived peptide CQ (amino acids 37 to 72; SEQ ID NO:4), RE (amino acids 49 to 86; SEQ ID NO:5), and Tat-2-derived peptide CP (amino acids 66 to 97; SEQ ID NO:6) was entrusted to TANA Lab. L.C. (Texas, USA).

The aptamer RNA^{Tat}oligonucleotide (aptamer RNA, SEQ ID NO:1) shown in Fig. 1 was synthesized with an RNA/DNA synthesizer (Applied Biosystem model 394) using phosphoramidites (Glen Corporation, U.S.) and was deprotected and purified according to a known method described by the present inventors (Yamamoto, R., Murakami, K., Taira, K. and Kumar, P.K.R., *Gene Ther. Mol. Biol.* **1**, 451-466 (1998)).

On the other hand, oligonucleotide chains corresponding to the 5'-sid e of the modulate aptamer of the present invention (DA-1, DA-3, DA-5, and DA-7; SEQ ID NOS: 7, 8, 9, and 10, respectively), and oligonucleotide chains corres ponding to the 3'-side of said aptamer (DA-2, DA-4, DA-6, and DA-8, SEQ ID NOS: 11, 12, 13, and 14, respectively) were respectively synthesized and deprotec ted in the same manner as the above aptamer RNA. The 5'-chains of the modula te RNAs were γ-^{32}P-ATP-labeled with T4 polynucleotide kinase.

Example 2:

Gel shift assay

Using a gel shift assay, 5 types of modulate aptamers were examined for their conjugate formation.

Double strand formation of RNA oligonucleotides was analyzed in the presence of Tat or Tat-derived peptides, CQ, RE, or CP by a previously reported method (Yamamoto, R., Murakami, K., Taira, K. and Kumar, P.K.R., *Gene Ther. Mol. Biol.* **1**, 451-466 (1998)).

Eight RNA oligonucleotides having the potential to form 5 types of double strand were analyzed (Fig. 2). In all cases, the 5'-chains of the double-stranded aptamer RNAs (DA-1,DA-3,DA-5,DA-7; SEQ ID NO:7, 8, 9, and 10, respectively) were γ -^{32}P-ATP-labeled. In 10μl of Tat-binding buffer (10 mM Tris-HCl, pH7.8, 70 mM NaCl, 2 mM EDTA, 0.01% Nonidet P-40), 5'-end labeled RNA (2 kcpm) and 200 nM of an unlabeled complementary chain RNA (relative to DA-1: DA-2 or DA-4; relative to DA-3: DA-4; relative to DA-5: DA-6; relative to DA-7: DA-8) were mixed in the presence of 40 nM *E. coli* tRNA.

To this was added 20 nM of CQ peptide or 200 nM of Tat protein, and the mixture was allowed to stand for 1 hour at 30℃. The reaction product was separated on non-denaturing polyacrylamide gel (15%), and the amount of conjugate formation in the presence or in the absence of the protein or peptide was measured with an image analyzer (BAS2000, Fujifilm, Japan).

Results are shown in Figure 3. Among the five types of oligonucleotide pairs, four pairs: DA-1/DA-2, DA-3/DA-4, DA-5/DA-6, and DA-1/DA-4 (i, ii, iii, and v, respectively, in Figs. 2 & 3), formed conjugates with high affinity in the presence of Tat protein (200 nM, lane 4) or Tat-derived peptide CQ (20 nM, lane 3) and did not form conjugates in the absence of Tat protein (lane 2). In particular, DA-5/DA-6 oligonucleotide pair formed a conjugate in the presence of Tat protein or CQ peptide more efficiently than the others, with conjugates with Tat at 50% and conjugates with CQ at 84% (Fig. 3iii).

Example 3:

Kinetic Analysis

The equilibrium dissociation constant (Kd) for conjugates of modulate aptamer RNA oligonucleotides DA-1/DA-2 (Fig. 2i) and DA-5/DA-6 (Fig. 2iii) obtained in Example 1, with CQ peptide was analyzed by gel shift assay in the presence of various concentrations of CQ (0.1 to 12.8 nM, 2 to 64 nM, respectively).

5'-end labeled RNA (50 pM) of DA-1 or DA-5, and RNA chains complementary thereto were mixed in a 10 μl Tat-binding buffer, and 40 nM tRNA was added as a non-specific competitor. CQ peptide (0.5 to 64 nM) was added and the mixture was allowed to stand for 1 hour at 30℃. The reaction product was separated on a non-denaturing polyacrylamide gel (15%), and analyzed. Values for B_{max} and Kd were determined from the following binding equation:

$$Y=B_{max} \times X/Kd+X$$

Y: Specific binding, B_{max}: maximum binding, X: ligand concentration

Non-linear regression analysis was performed with Graphpad PRISM software (Graphpad Software Inc, U.S.).

As is clear from the results shown in Figs. 4 and 5, Kd values for DA-5/DA-6-CQ, DA-1/DA-2-CQ conjugates were 0.5 nM and 400 nM, respectively, with the Kd value for the DA-1/DA-2-CQ conjugate being 800 times greater than that for the DA-5/DA-6-CQ conjugate. This difference is thought to be primarily caused by the two additional G-C base pair formations at both ends of the molecule in the DA-5/DA-6-CQ conjugate.

In other Tat peptides also, similar results were obtained. The modulate aptamers reconstituted a double-stranded RNA in the presence of Tat or CQ peptide, and exhibited affinity and specificity to the Tat protein comparable to that of hairpin aptamer RNA^{Tat} (Fig. 1 left).

Example 4

Comparison with Core Factor Mutant-1

From the fact that the Kd value for the DA-5/DA-6-CQ conjugate approximated with the Kd value of a hairpin aptamer RNA^{Tat}, it is thought that modulate aptamers, particularly DA-5/DA-6, reconstitute a binding core factor in the presence of Tat protein or CQ peptide. The binding core factor of aptamer RNA^{Tat} necessary for binding to the Tat protein consists of a central helix of four base pairs and two bulges comprising two residues each on each side thereof, and through site specific mutation experiments it was found that the U residues of both bulges were extremely important for binding to the Tat protein. Therefore, from kinetic analysis of the hairpin aptamer and modulate aptamer oligonucleotides DA-5/DA-6, these RNA compositions, i.e. both chains of DA-5 and DA-6 were predicted to interact with Tat protein.

In order to confirm this, the conjugate formation ability of a mutant (DA-5i/DA-6i (SEQ ID NO:15/16), DA-5i was labeled with γ -^{32}P-ATP) wherein the C residues of DA-5 and DA-6 were substituted by U residues (Figure 6A), was examined in the same manner as in Example 2. Results using CQ peptide are shown in Figure 6B. As is clear from the results of Figure 6B, this oligonucleotide pair (DA-5i/DA-6i) was unable to form a conjugate. This result indicated that functional groups important in the binding of the hairpin aptamer to Tat-1 (for example, the nitrogen at position 3 of a U residue), were also important in the binding of modulate aptamer DA-5/DA-6 to Tat-1.

Example 5

Comparison with Core Factor Mutant-2

In respect of a TAR-1 RNA-derived RNA oligonucleotide, DT-1/DT-2 (Fig. 7A, the sequence of DT-1 is represented by SEQ ID NO: 17) also, a comparison was made with DA-5/DA-6 in respect of binding to Tat-1 protein or CQ peptide in a manner similar to Example 2. Results using CQ peptide are shown in Fig. 7B. As is clear from the results in Fig. 7B, DT-1/DT-2 did not form a conjugate even in the presence of excess oligonucleotide (DT-2) and excess CQ.

From these results, it was clear that a modulate aptamer exhibits high

affinity due to repetition and arrangement in the opposite direction of the TAR-1 RNA core factor, and binds sequence specifically with Tat protein and CQ peptide.

Example 6

Binding specificity to target protein

In the presence of HCV NS3 protein which is an RNA binding protein having a protease domain and an RNA helicase domain, a gel shift binding assay was performed with modulate aptamer oligonucleotides (DA-1/DA-2, DA-3/DA-4, DA-5/DA-6, DA-7/DA-8, DA-1/DA-4). In the gel shift analysis, this NS3 protein did not form a conjugate with the modulate aptamer oligonucleotide of the present invention which targets HIV-1 Tat protein (Data not shown).

Example 7

Affinity with HIV-2 Tat protein

In Tat proteins of HIV-1 and HIV-2, the core regions thereof (from cystein 36 to proline 57 of Tat-2) exhibit approximately 65% homology. In addition, from the research of the present inventors, it was found that an aptamer binds to Tat-2 peptide (CP, amino acids 66 to 97, SEQ ID NO:6) with greater affinity than TAR-2 RNA, and this fact indicated that the RNA binding characteristic of the two proteins was similar. Fig. 8 indicates that although efficiency is lower when compared with Tat-1 peptides CQ, RE, Tat-2 peptide CP also promotes formation of a double-strand of modulate aptamer DA-5/DA-6, respectively.

Example 8

Analyte (Tat)-dependent hybridizing oligonucleotide assay

The modulate aptamer of the present invention has potential as a tool for use in detection of a target protein such as Tat protein. A diagnostic assay such as indicated in Fig. 9 was developed and tested. In this analyte-dependent hybridizing oligonucleotide assay (analyte-dependent hybridizing oligonucleotide assay, ADHONA),

a 3'-fluorescein oligonucleotide (DA-9, SEQ ID NO:18), a 3'-biotin oligonucleotide (DA-10, SEQ ID NO:19), and a streptoavidin-coated microtiter plate were used (Fig. 9A and B).

The streptoavidin-coated microtiter plate was purchased from Labsystems of Finland. 3'-biotinized oligonucleotide DA-10 (5 pmol) was placed in a streptoavidin plate (well), and left to stand at room temperature for 10 minutes in a 50 μl of Tat binding buffer, and after allowing to bind with streptoavidin, the plate was washed with 200 μl of Tat binding buffer to wash away unbound oligonucleotides. Next, 50 μl of Tat binding buffer containing 10 pmol of 3'-fluorescein DA-9 and Tat-1 or Tat peptide (CQ or CP) was added, and the plate was allowed to stand for 30 minutes at 30℃, and finally washed again with 200 μ l Tat binding buffer to remove unbound substances. Fluorescence was analyzed with a fluoro image analyzer (FluorImager595). Excitation was at a wavelength of 488 nm, and detection at 530 nm. As a control, the same operations were performed in the absence of Tat-1 protein and Tat peptide.

Results are shown in Figs. 10 and 11. In the presence of 10 to 100 pmol of CQ peptide, fluorescence intensity increased to 500 to 2500 times that of the control (Fig. 10). Further, using Tat-1 protein (200 pmol), a fluorescence signal equivalent to that obtained with 10 pmol CQ peptide, was obtained. (Fig.11).

As is clear from the above results, conjugates were detected only in the presence of target protein, CQ peptide or Tat-1 protein, and the presence of the target protein was detected by using the modulate aptamer of the present invention.

Example 9

Reaction with co-existent Mammal cell nuclear extract

In order to be a diagnostic tool having good efficiency, in the assay sho wn in Fig. 9, it must be able to exhibit a quantitative result even in the presenc e of a crude sample such as a mammal cell nuclear extract. A crude sample may contain proteins and other mixtures which will interfere with the assay, and may further include proteins that promote annealing of a complementary sequence (Po

rtman, D.S. and Dreyfuss, G., *EMBO J.* **13**, 213-221 (1994)). Here, the above ass ay was performed in the presence of DA-9/DA-10 with 8 units HeLa nuclear extr act (Promega, U.S.), and 40 units of RNase inhibitor (TOYOBO, Japan). Results are shown in Fig. 12. In the case where only HeLa nuclear extract was present, t he modulate aptamer oligonucleotide did not form a double strand, and did not re main on the microtiter well (Even with 40 units of RNase inhibitor, a small amo unt of RNase activity remained).

This result indicated that the assay of the present invention was suitable for detecting Tat protein in the presence of mammalian cell nuclear extracts such as those in infected cells and the like.

Example 10

Use as a molecular beacon aptamer

As described above, formation of a stable double-stranded structure of aptamer-derived oligonucleotides such as DA-5 and DA-6 was able to be efficiently regulated by Tat or a Tat-derived peptide. Using this, the present invention was first able to provide a method for detecting Tat or a peptide thereof using a molecular beacon aptamer.

Based on a sequence of a modulate aptamer, a quencher substance [4'-(4'-dimethyl-aminophenyl-azo) benzoic acid (DABSYL)] was connected by a covalent bond to the 5'-end, and a fluorescent substance, fluorescein, was connected to the 3'-end of an oligonucleotide chain (SEQ ID NO:20) which has mutually complementary nucleotide sequences of 6 consecutive nucleotides within the molecule, which independently adopts a stem-loop structure and which can form a modulate aptamer by pairing with DA-6, thereby constructing a molecular beacon aptamer which was then synthesized (DA13(C-A), Fig. 13 left). Specifically, using phosphoramidite (Glen Corporation, U.S.), the oligonucleotide was synthesized with a RNA/DNA synthesizer (Applied Biosystem Model 394) and after 5'-fluoresceination and 3'-DABSYLation, deprotection was carried out according to methods established in the

art. This complementary pair formation between DA13 (C-A) and DA-6 is indicated in Fig. 13. On the other hand, for the purpose of evaluating relative amounts of fluorescence intensity when the fluorescent substance and quencher substance are separated by a distance, DA-13C (SEQ ID NO:21) which has a sequence completely complementary to DA-13 (C-A) was synthesized using phosphoramidite (Glen Corporation, U.S.), with an RNA/DNA synthesizer (Applied Biosystem model 394) (Fig. 13 right).

Detection of Tat or a Tat peptide using a molecular beacon aptamer was performed as follows:

In a 0.5 ml tube, 10 nM of DA-13 (C-A) and 100 nM of DA-6 were mixed in 50 μl of Tat binding buffer (10 mM Tris-HCl, pH 7.8, 70 mM NaCl, 2 mM EDTA, 0.01% Nonidet P-40) containing 40 nM of *E. Coli* tRNA, in the presence or absence of 100 nM of CQ peptide, and allowed to stand for 30 minutes at 30℃. The fluorescence intensity emitted as a result was measured with FluorImager (Molecular Dynamics). As a comparison, fluorescence intensity was measured under similar conditions in respect of 10 nM of DA-13 (C-A) only, or 10 nM DA-13 (C-A)/100 nM DA-13C.

As shown in the results in Figs. 14 and 15, DA-13 (C-A) itself adopted the stem-loop structure shown in Fig. 13 - left, and fluorescence was hardly detected. When both DA-6, and CQ being the target protein were present, this stem-loop structure underwent a structural change to a double-stranded structure, and fluorescence intensity increased dramatically. In the absence of DA-6 oligo or CQ, DA-13 (C-A) oligo did not undergo this structural change. On the other hand, DA-13 (C-A) oligonucleotide and DA-13C oligonucleotide formed a conjugate in Tat binding buffer even in the absence of CQ, and the fluorescence intensity thereof exhibit the maximum value. This was thought to be most likely due to the fluorescent substance binding portion and the quencher substance binding portion of DA-13(C-A)/DA-13C being separated by a distance 25 base pairs when compared with DA-13 (C-A)/DA-6 conjugate; and the DA-13 (C-A)/DA-13C conjugate being more stable than the DA-13 (C-A)/DA-6 conjugate.

As is clear from the above results, from the fact that fluorescent intensity increased markedly in the presence of DA-6 and CQ, it is apparent that molecular beacon [DA-13 (C-A)/DA-6] undergoes a structural change in the presence of the target protein: Tat or Tat peptide, and Tat can be efficiently detected by monitoring this structural change through fluorescence measurement.

INDUSTRIAL APPLICABILITY

The modulate aptamer of the present invention has the following numerous advantages when compared to known conventional long non-modulate aptamers.

1) Short RNA oligonucleotides can be synthesized with higher efficiency than long ones.

2) Modification for stabilizing nucleic acids may be partial in the aptamer.

3) Low cost.

4) Suitable structuring of the modulate aptamer is promoted by the analyte.

Further, the above results indicate that the method of detecting a protein with the modulate aptamer of the present invention has high sensitivity and high specificity, and can be easily applied to nucleic acid array analysis techniques having complex steps. Further, when the RNA aptamer of the present invention is completely protected against ribonuclease, the method will be even more effective. The method of the present invention can detect not only HIV Tat protein, but can be generally applied to the detection of other proteins such as HIV Rev protein using a modulate aptamer for RRE (Rev response element) by selecting suitable non-modulate and modulate-type aptamers from a combined library.

Further, in respect also of the molecular beacon aptamer which is one embodiment of the modulate aptamer of the present invention, it is possible to make modification such that the aptamers have resistance against nuclease, thereby allowing these stabilized molecular beacon aptamers to be applied for the detection of Tat protein in living cells (for example, in HIV infected cells).

All publications, patent and patent applications cited herein are incorporated herein in their entirety by reference.

CLAIMS

1. A modulate aptamer being an aptamer constituted by two complementary oligonucleotide chains, which forms a conjugate and stabilizes only in the presence of a target protein.

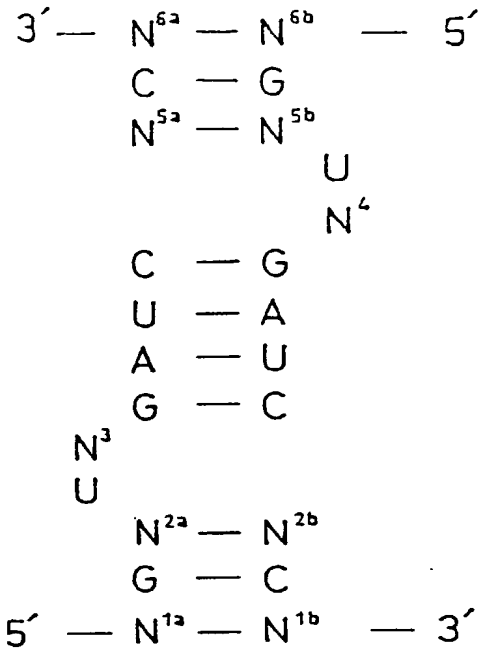
2. The modulate aptamer according to claim 1, wherein one or both of the two oligonucleotide chains is radioactively or non-radioactively labeled.

3. The modulate aptamer according to claim 1, wherein one of the oligonucleotide chains constituting the modulate aptamer has intramolecularly, mutually complementary sequences of four or more consecutive nucleotides and has a stem-loop structure in the absence of a target protein.

4. The modulate aptamer according to claim 3, wherein a fluorescent substance is bound to the 5' or 3'-end of the oligonucleotide of the stem-loop structure, and a quencher substance for the fluorescent substance is bound to the 3' or 5' end thereof, respectively.

5. The modulate aptamer according to any one of claims 1 to 4, wherein the target protein is HIV-1 Tat protein and/or a fragment thereof.

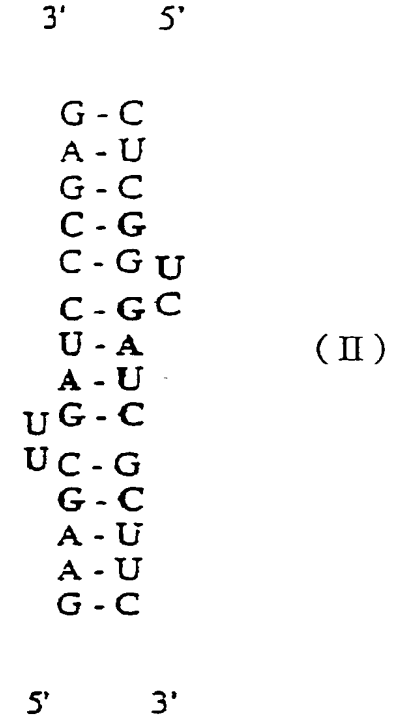
6. The modulate aptamer according to claim 5, which comprises the nucleotide sequence represented by the following secondary structure (I):



(I)

(In the structure, N^{1a} and N^{1b} represent at least 1 pair of nucleobases capable of complementary base pair formation; N^{2a} and N^{2b} represent at least 1 pair of nucleobases capable of complementary base pair formation; N^3 and N^4 each independently represent 1 or 2 nucleobases; N^{5a} and N^{5b} represent at least 1 pair of nucleobases capable of complementary base pair formation; N^{6a} and N^{6b} represent at least 1 pair of nucleobases capable of complementary base pair formation; and solid lines represent hydrogen bonds between nucleobases.)

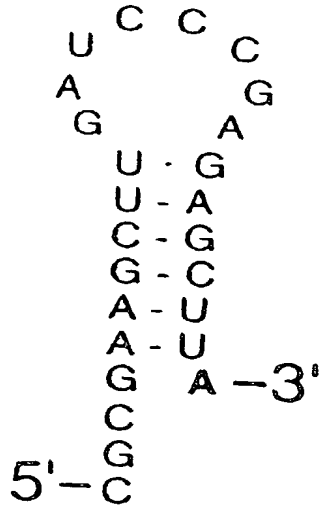
7. The modulate aptamer according to claim 6, which comprises the nucleotide sequence represented by the following secondary structure:



(II)

(In the structure, solid lines represent hydrogen bonds between nucleobases.)

8. The modulate aptamer according to claim 3, wherein one of the oligonucleotide chains constituting the modulate aptamer has a nucleotide sequence represented by the following secondary structure (III):



(III)

(In the structure, solid lines represent hydrogen bonds between nucleobases.)

9. A method of detecting a target protein, which comprises radioactively or non-radioactively labeling one oligonucleotide chain of the modulate aptamer according to claim 1, and detecting the presence and/or amount of the target protein with a conjugate formed in the presence of the target protein as an indicator.

10. A method of detecting a target protein, which comprises immobilizing one oligonucleotide chain of the modulate aptamer according to claim 1 on a support, and detecting the presence and/or amount of a target protein with a conjugate formed by addition of the other oligonucleotide labeled radioactively or non-radioactively as an indicator.

11. The method according to claim 9 or 10, wherein the oligonucleotide chain of the stem-loop structure according to claim 4 is used as the oligonucleotide chain labeled.

12. The method according to claim 10 or 11, wherein the immobilization is by specific binding between avidin or streptoavidin and biotin.

13. The method according to claim 9 or 10, wherein the non-radioactive label is fluorescein and the conjugate is detected by the fluorescent signal thereof.

14. The method according to any one of claims 9 to 13, wherein the target protein is an HIV-1 Tat protein and/or a fragment thereof.

15. A kit for detecting a target protein, which comprises the following (a) to (c):

(a) a support,

(b) one of the oligonucleotide chains of the modulate aptamer according to claim 1 to be immobilized on the support,

(c) the other oligonucleotide chain which is radioactively or non-radioactively labeled and forms a conjugate in the presence of a target protein.

16. The kit for detecting a target protein according to claim 15, wherein the target protein is an HIV-1 Tat protein and/or a fragment thereof.

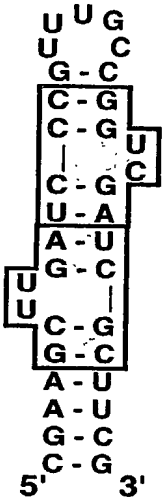
17. The kit for detecting a target protein according to claim 15, wherein one of the oligonucleotide chain (b) is the 5'-chain or 3'-chain of the modulate aptamer according to claim 6 or 7, and the other oligonucleotide chain (c) is the 3'-chain or 5'chain, respectively.

18. The kit for detecting a target protein according to claim 15 or 16, wherein the oligonucleotide chain of the stem-loop structure according to claim 4 is used as the labeled oligonucleotide chain.

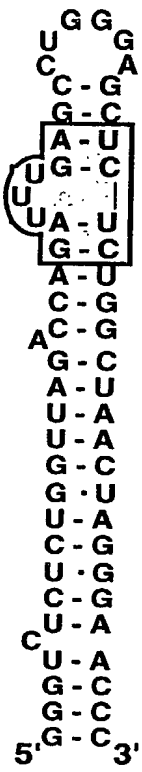
ABSTRACT

The object of the present invention is the development of a novel strategy for providing an effective biosensor which forms a conjugate and stabilizes only in the presence of a target protein or substance to be analyzed. That is, the present invention provides a modulate aptamer which specifically binds with a specific target protein, particularly a human immunodeficiency virus type-1 (HIV-1) Tat protein, and a method and kit for detecting the target protein, in particular, HIV-1 Tat protein using the modulate aptamer.

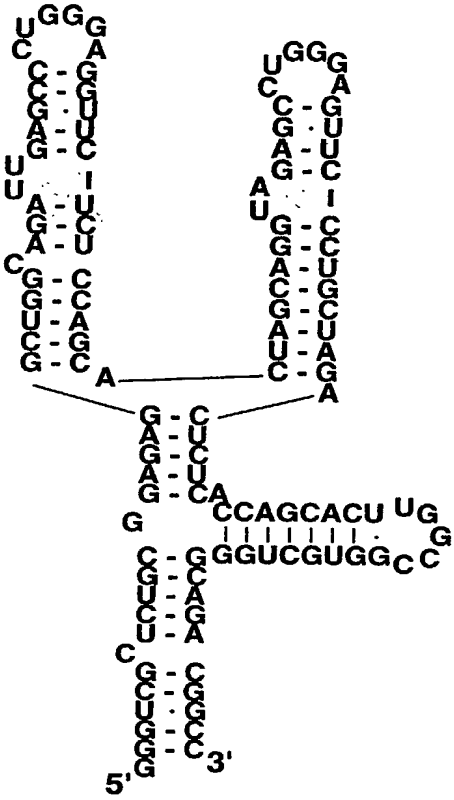
FIG.1



Aptamer RNA^{Tat}

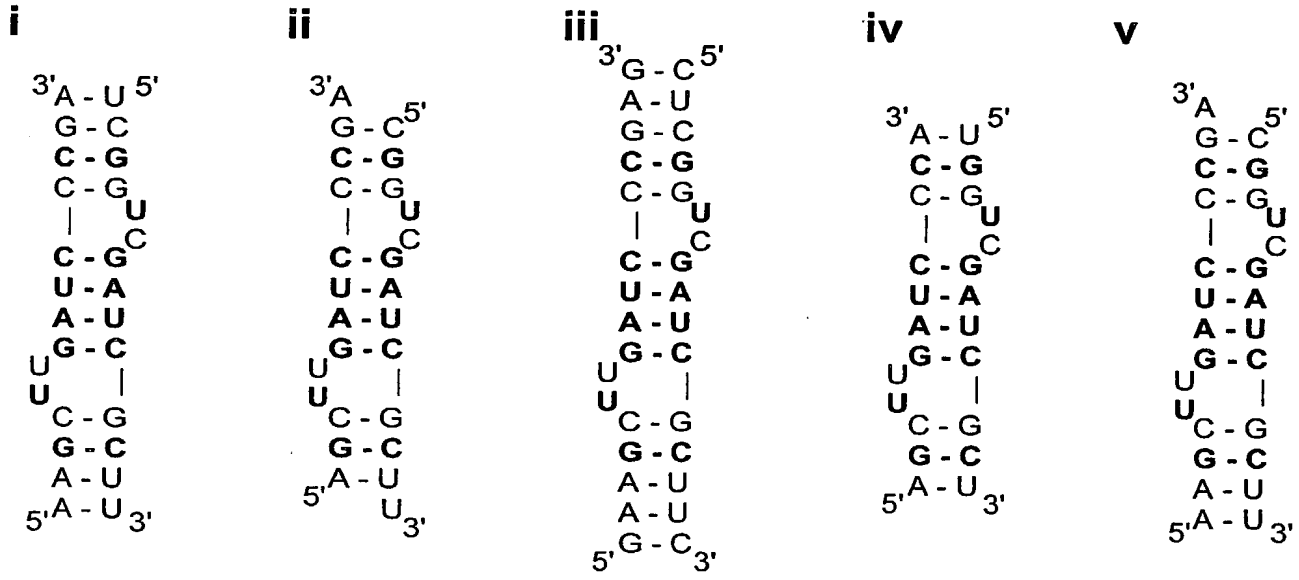


TAR-1 RNA



TAR-2 RNA

FIG.2



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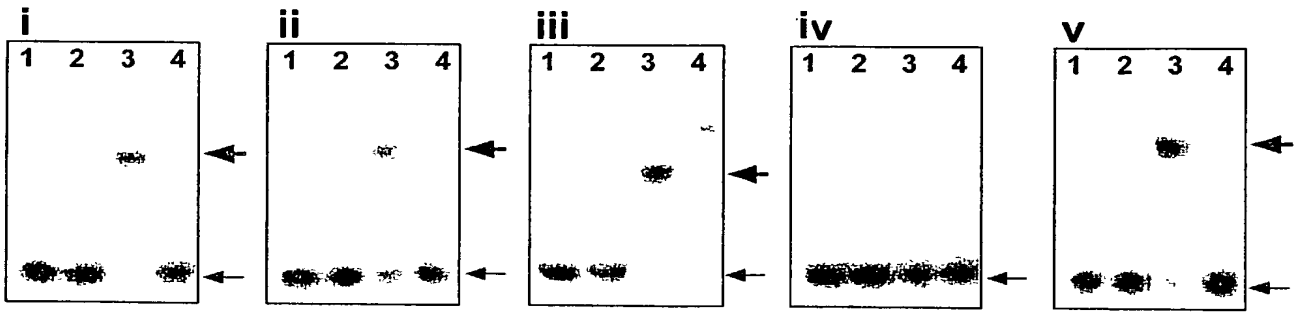
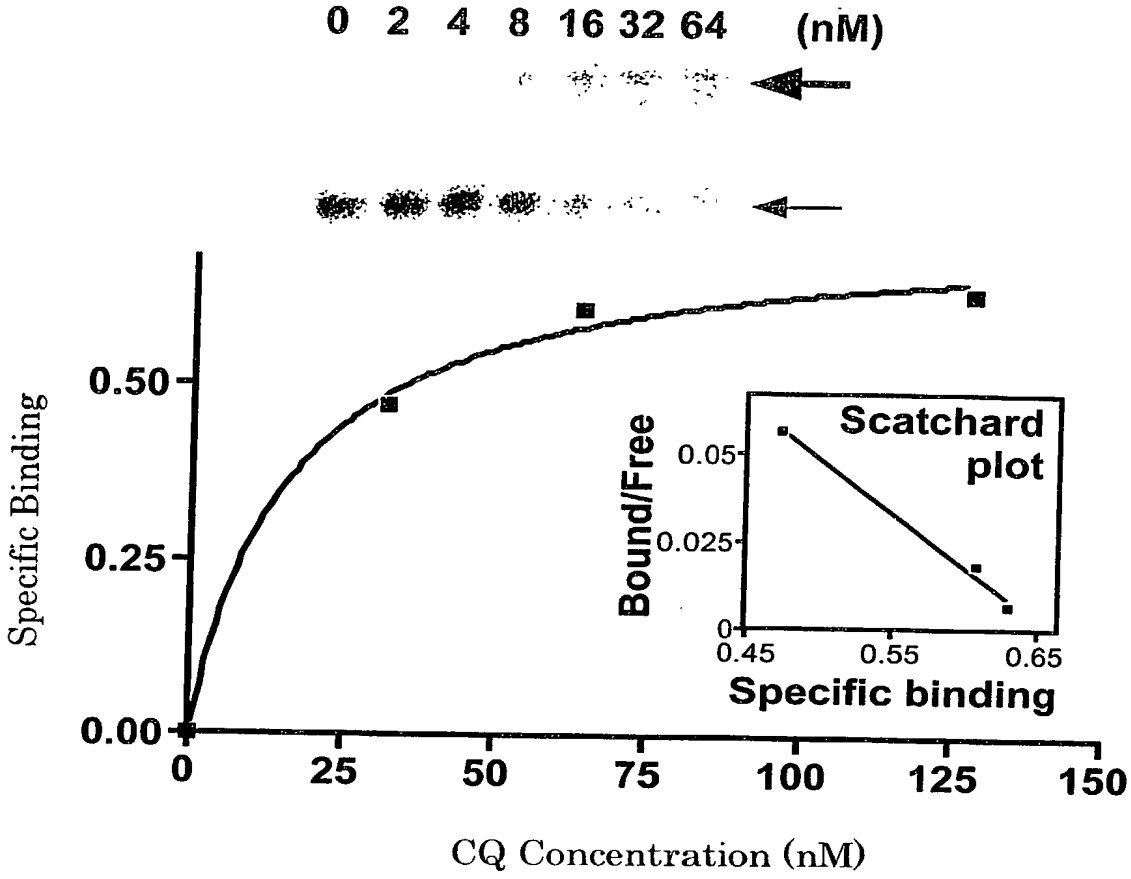


FIG.4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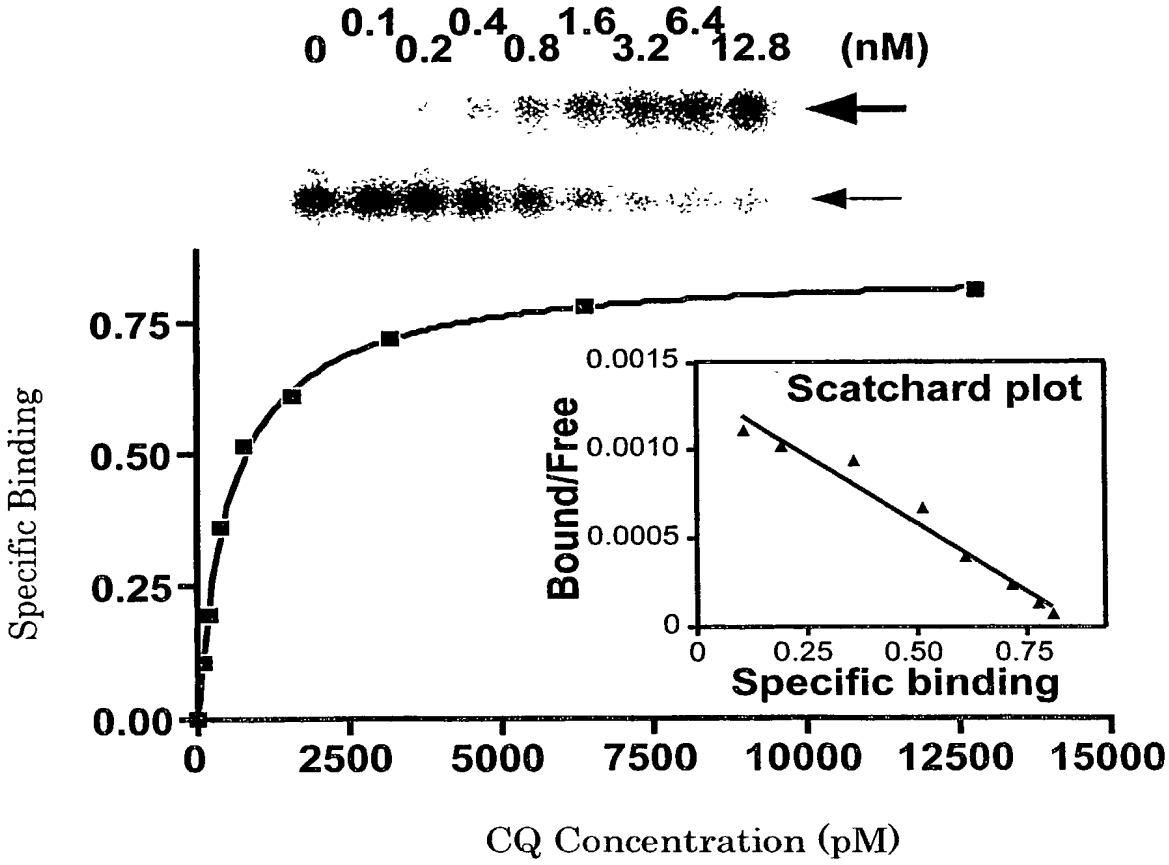
Specific Binding
0.50
0.25
0.00
0
25
50
75
100
125
150
CQ Concentration (nM)
Bound/Free
0.05
0.025
0
0.45
0.55
0.65
Specific binding
Scatchard plot

FIG.5


0 0.1 0.2 0.4 0.8 1.6 3.2 6.4 12.8 (nM)
Specific Binding
0.75
0.50
0.25
0.00
0
2500
5000
7500
10000
12500
15000
CQ Concentration (pM)
Scatchard plot
Bound/Free
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0.0010
0.0005
0
0.25
0.50
0.75
Specific binding

FIG.6

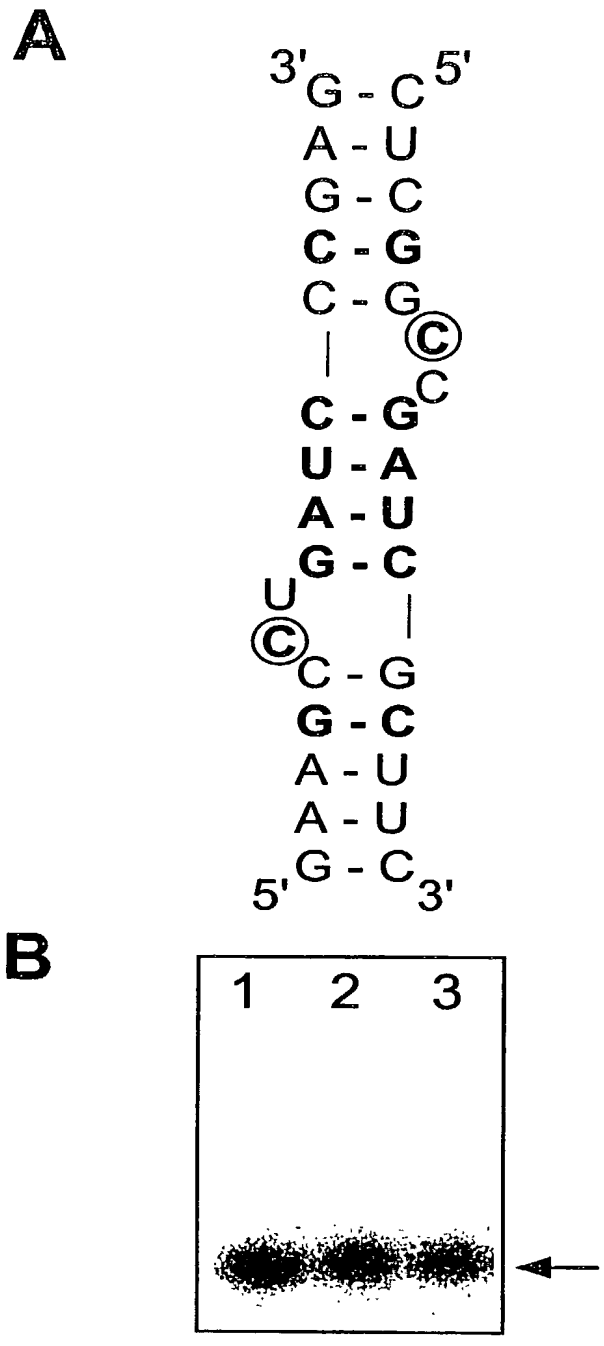

FIG.7

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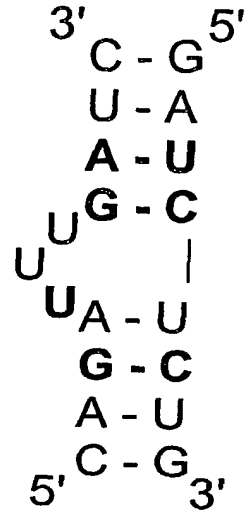


B

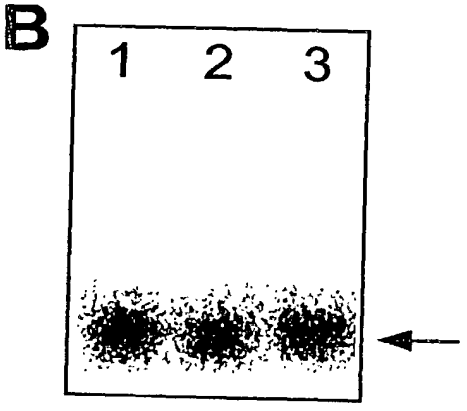


FIG.8

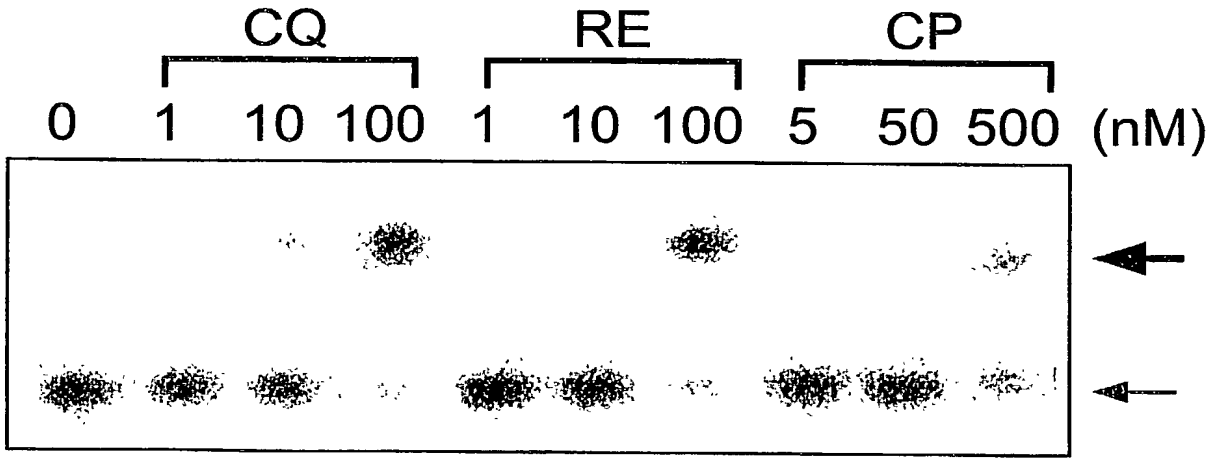


FIG.9

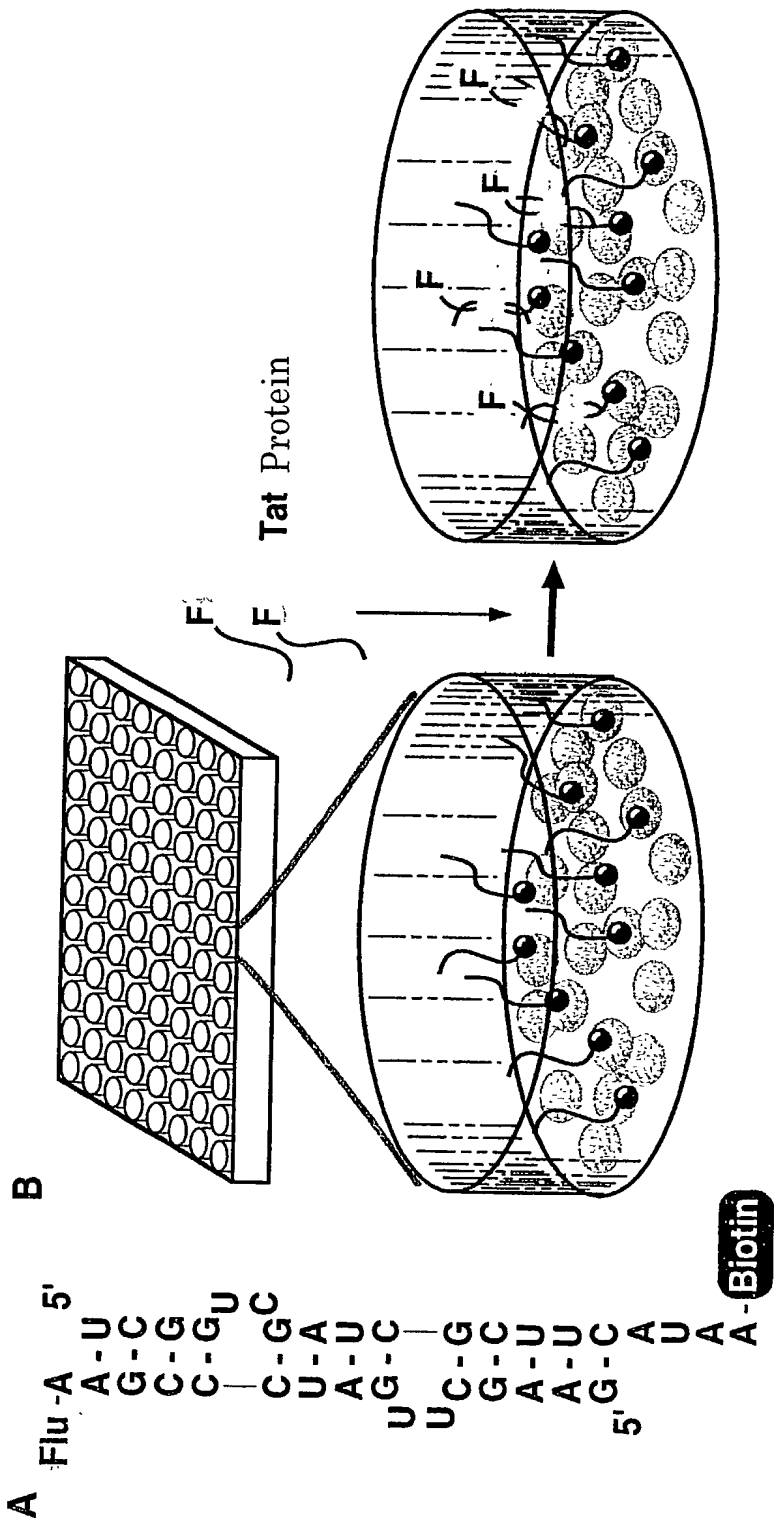

A
Flu-A
5'
A-U
G-C
C-G
C-G
U
C-G
C
U-A
A-U
C
A-U
C-G
C-G
U
G-C
A-U
A-U
G-C
A
U
A
A-Biotin
5'
B
F
F
Tat Protein
F
F
F
F

FIG.10

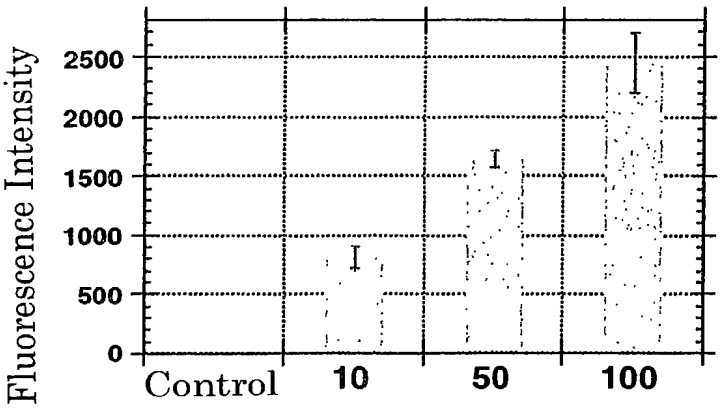


FIG.11

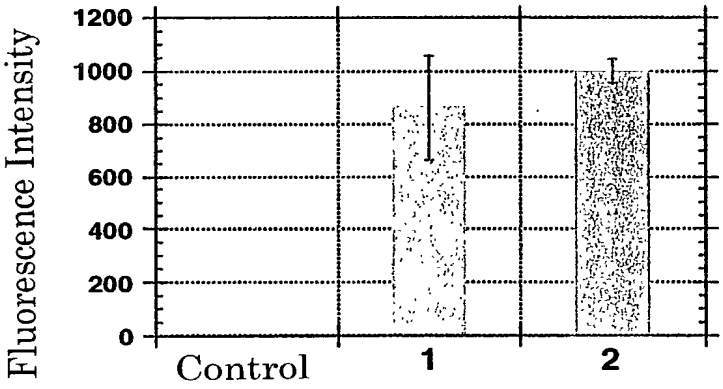


FIG.12

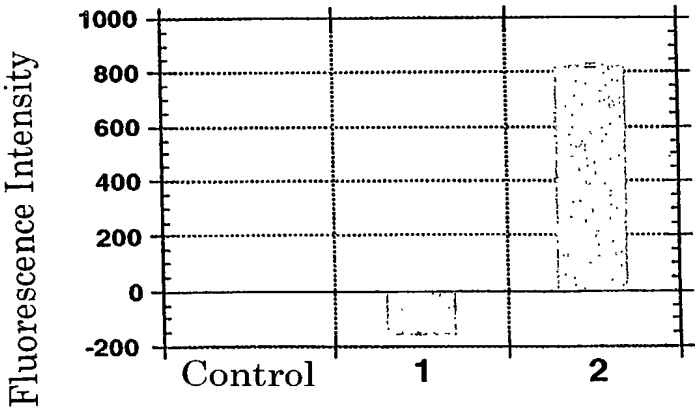


FIG.13

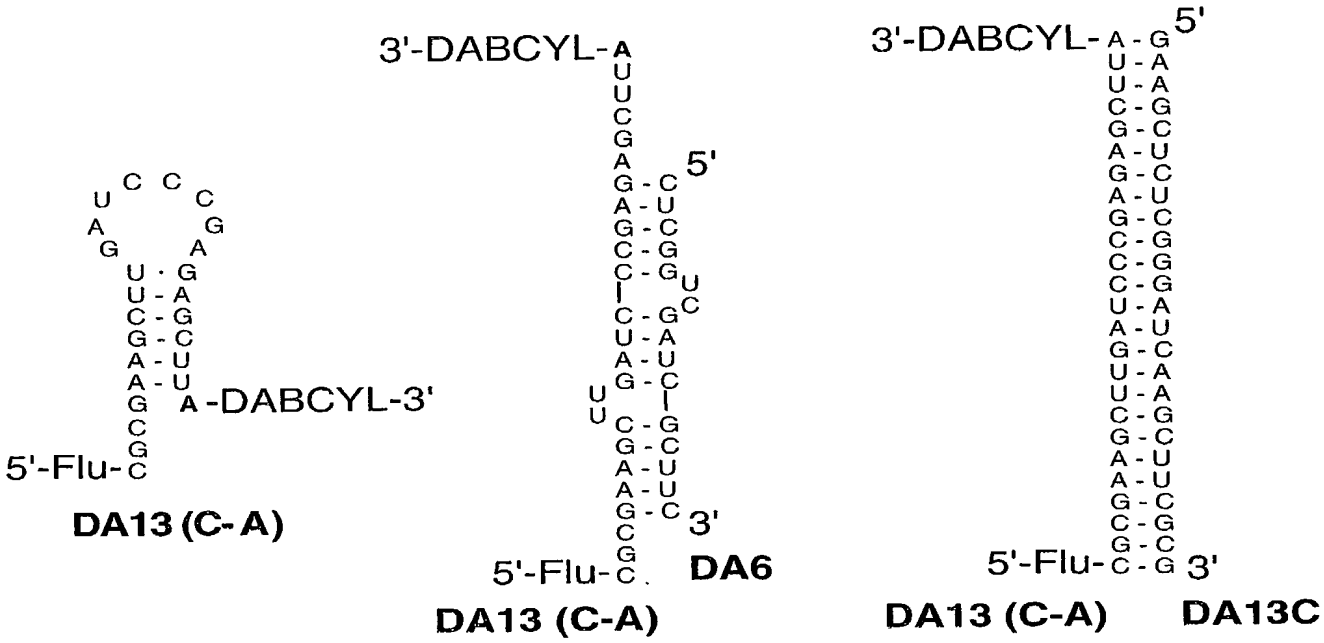


FIG.14

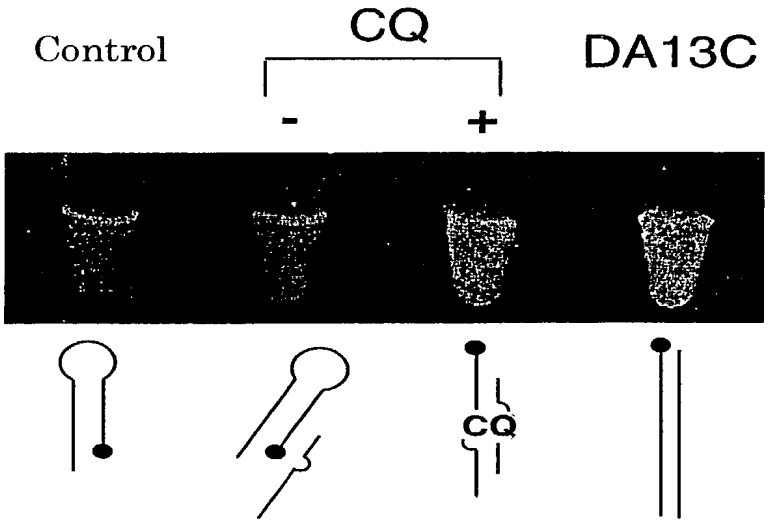
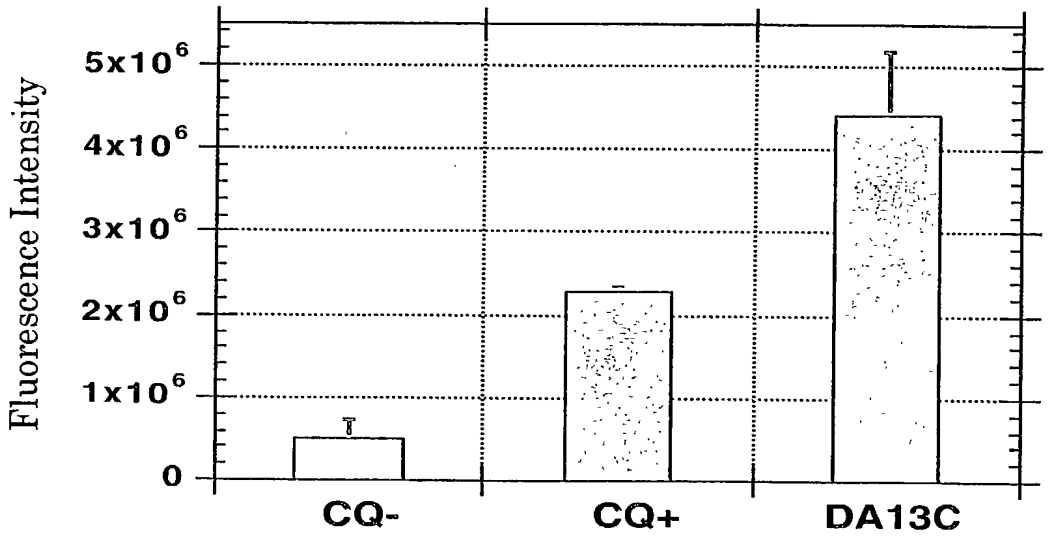


FIG.15



Attorney s Docket No.:_____

DECLARATION, POWER OF ATTORNEY AND PETITION

I (We), the undersigned inventor(s), hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I (We) believe that I am (we are) the original, first, and joint (sole) inventor(s) of the subject matter which is claimed and for which a patent is sought on the invention entitled

MODULATE APTAMER AND METHOD OF DETECTING TARGET PROTEIN BY USING THE SAME

the specification of which

- ☐ is attached hereto.
- ☐ was filed on _____ as Application Serial No. _____ and amended on _____.
- ■ was filed as PCT international application Number PCT/JP00/01969 on March 29, 2000, and was amended under PCT Article 19 on _____ (if applicable).

I (We) hereby state that I (We) have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above; that I (We) do not know and do not believe that this invention was ever known or used before my invention or discovery thereof, or patented or described in any printed publication in any country before my invention or discovery thereof, or more than one year prior to this application, or in public use or on sale in the United States for more than one year prior to this application; that this invention or discovery has not been patented or made the subject of an inventor s certificate in any country foreign to the United States on an application filed by me or my legal representatives or assigns more than twelve months before this application.

I (We) acknowledge the duty to disclose information known to be material to the patentability of this application as defined in Section 1.56 of Title 37 Code of Federal Regulations.

I (We) hereby claim foreign priority benefits under Section 119(a)-(d) of Title 35 United States Code, of any foreign application(s) for patent or inventor s certificate listed below and have also identified below any foreign application for patent or inventor s certificate having a filing date before that of the application on which priority is claimed:

Application No.	Country	Filing date	Priority claimed	
288677/1999	Japan	October 8, 1999	■ Yes	☐ No
			☐ Yes	☐ No
			☐ Yes	☐ No
			☐ Yes	☐ No

I hereby claim the benefit under Section 119(e) of Title 35 United States Code, of any United States application(s) listed below.

(Application Number)	(Filing Date)
(Application Number)	(Filing Date)

I (We) hereby claim the benefit under Section 120 of Title 35 United States Code, of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Section 112 of Title 35 United States Code, I (We) acknowledge the duty to disclose material information as defined in Section 1.56(a) of Title 37 Code of Federal Regulations, which occurred between the filing date of the prior application and national or PCT international filing date of this application:

Application Serial No.	Filing Date	Status (pending, patented, abandoned)

And I (We) hereby appoint: Bing Ai, Registration No. 43,312; Richard J. Anderson, Registration No. 36,732; James W. Babineau, Registration No. 42,276; Joseph R. Baker, Registration No. 40,900; Ingrid A. Beattie, Registration No. 42,306; Robert M. Bedgood, Registration No. 43,488; Christine P. Bellon, Registration No. 41,611; William E. Booth, Registration No. 28,933; Samuel Borodach, Registration No. 38,388; Roger S. Borovoy, Registration No. 20,193; Charles J. Boudreau, Registration No. 42,350; John A. Burtis, Registration No. 39,924; Elizabeth Chien-Hale, Registration No. 44077; Ruffin B. Cordell, Registration No. 33,487; John D. Cowart, Registration No. 38,415; Gary L. Creason, Registration No. 34,310; Lee Crews, Registration No. 43,567; Andrew T. D'Amico, Jr., Registration No. 33,375; Sean P. Daley, Registration No. 40,978; Peter J. Devlin, Registration No. 31,753; John A. Dragseth, Registration No. 42,497; William J. Egan, III, Registration No. 28,411; Mark S. Ellinger, Registration No. 34,812; Eldora L. Ellison, Registration No. 39,967; J. Peter Fasse, Registration No. 32,983; David L. Feigenbaum, Registration No. 30,378; Richard P. Ferrara, Registration No. 30,632; Stephan J. Filipek, Registration No. 33,384; J. Patrick Finn III, Registration No. 44,109; Janis K. Fraser, Registration No. 34,819; John W. Freeman, Registration No. 29,066; Timothy A. French, Registration No. 30,175; John J. Gagel, Registration No. 33,499; Edouard A. Garcia, Registration No. 38,461; Diane L. Gardner, Registration No. 36,518; Kurt L. Glitzenstein, Registration No. 39,686; David J. Goren, Registration No. 34,609; Robert A. Greenberg, Registration No. P-44,133; James A. Gromada, Registration No. P-44,727; H. Sanders Gwin, Jr., Registration No. 33,242; William D. Hare, Registration No. 32,030; Scott C. Harris, Registration No. 32,030; John F. Hayden, Registration No. 37,640; Mark J. Hebert, Registration No. 31,766; George E. Heibel, Registration No. 42,648; Gilbert H. Hennessey, Registration No. 25,759; Charles Hieken, Registration No. 18,411; Robert E. Hillman, Registration No. 22,837; William J. Hone, Registration No. 26,739; Shane H. Hunter, Registration No. 41,858; Allison A. Johnson, Registration No. 36,173; Mark D. Kirkland, Registration No. 40,048; Lawrence K. Kolodney, Registration No. 43,807; Linda Liu Kordziel, Registration No. 39,732; Phyllis K. Kristal, Registration No. 38,524; John Land, Registration No. 29,554; Celia H. Leber, Registration No. 33,524; G. Roger Lee, Registration No. 28,963; Samuel S. Lee, Registration No. 42,791; Monica L. Lewis, Registration No. 42,600; John T. Li, Registration No. 44,210; Ronald C. Lundquist, Registration No. 37,875; Stuart Macphail, Registration No. 44,217; Gregory A. Madera, Registration No. 28,878; Denis G. Maloney, Registration No. 29,670; Christopher S. Marchese, Registration No. 37,177; John F. McCabe, Registration No. 42,854; Monica McCormick Graham, Registration No. 42,600; Anita L. Meiklejohn,

Registration No. 35,283; Todd G. Miller, Registration No. 42,003; Ralph A. Mittelberger, Registration No. 33,195; Chris T. Mizumoto, Registration No. 42,899; James E. Mrose, Registration No. 33,264; Louis Myers, Registration No. 35,965; Robert C. Nabinger, Registration No. 33,431; Greg O'Bradovich, Registration No. 42,945; Frank R. Occhiuti, Registration No. 35,306; George C. Pappas, Registration No. 35,065; Andrew N. Parfomak, Registration No. 32,431; John B. Pegram, Registration No. 25,198; John C. Phillips, Registration No. 35,322; Frank P. Porcelli, Registration No. 27,374; Timothy A. Porter, Registration No. 41,258; Jon M. Powers, Registration No. 43,868; Eric L. Prahl, Registration No. 32,590; Paul A. Pysher, Registration No. 40,780; Frederick H. Rabin, Registration No. 24,488; J. Robin Rohlicek, Registration No. 43,349; Mathias W. Samuel, Registration No. 39,823; Stephen R. Schaefer, Registration No. 37,927; Richard M. Sharkansky, Registration No. 25,800; Steven J. Shumaker, Registration No. 36,275; Robert J. Silverman, Registration No. 42,149; John M. Skenyon, Registration No. 27,468; Jack L. Slobodin, Registration No. 29,110; Alan D. Smith, Registration No. 32,005; Jeffrey L. Snow, Registration No. 39,037; Reginald J. Suyat, Registration No. 28,172; Rene D. Tegtmeyer, Registration No. 33,567; Bao Q. Tran, Registration No. 37,955; Hans R. Troesch, Registration No. 36,950; Y. Rocky Tsao, Registration No. 34,053; Natasha C. Us, Registration No. P-44,381; Jonathan J Wainer, Registration No. 36,712; Gary A. Walpert, Registration No. 26,098; John R. Wetherell, Jr., Registration No. 31,678; Dorothy P. Whelan, Registration No. 33,814; Wayne E. Willenberg, Registration No. 28,488; John N. Williams, Registration No. 18,948; Charles C. Winchester, Registration No. 21,040.

110

I(We) hereby request that all correspondence regarding this application be sent to the firm of FISH & RICHARDSON P.C. whose Post office address is: 45 Rockefeller Plaza, Suite 2800, New York, New York 10111 U.S.A.

I (We) declare further that all statements made herein of my (our) knowledge are true and that all statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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Signature of Inventor

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NAME OF THIRD JOINT INVENTOR

Signature of Inventor

Date

Residence:

Citizen of:
Post Office Address:

Attorney's Docket No.: 11283-020US1 / PH-933PCT-US

#4

PTO/PCT Rec'd 25 JUL 2002

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant : Penmetcha Kumar et al.
Serial No. : 10/089,212
Filed : March 26, 2002
Title : MODULATE APTAMER AND METHOD OF DETECTING TARGET PROTEIN BY USING THE SAME

BOX PCT
U.S. Patent and Trademark Office
P.O. Box 2327
Arlington, VA 22202

VERIFIED STATEMENT UNDER 37 CFR §1.821(f)

I, Jennifer H. Payne, declare that I personally prepared the paper and the computer-readable copy of the Sequence Listing filed herewith for the above-identified application and that the content of both is the same.

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of The United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: July 5, 2002

Jennifer H. Payne

Fish & Richardson P.C.
225 Franklin Street
Boston, Massachusetts 02110-2804
(617) 542-5070 telephone
(617) 542-8906 facsimile

20456525 doc

CERTIFICATE OF MAILING BY EXPRESS MAIL

Express Mail Label No EF353816385US

I hereby certify under 37 CFR §1 10 that this correspondence is being deposited with the United States Postal Service as Express Mail Post Office to Addressee with sufficient postage on the date indicated below and is addressed to the Commissioner for Patents, P.O. Box 2327, Arlington, VA 22202.

July 25, 2002
Date of Deposit

Signature

Francisco Robles
Typed or Printed Name of Person Signing Certificate

SEQUENCE LISTING

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<120> Modulate aptamers and the method of detecting target proteins using them

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20 25 30

Leu Ser Lys Gln
35

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1 5 10 15

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Pro Thr Gly Pro Lys Glu
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1 5 10 15

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<400> 18

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<210> 19

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<212> RNA

<213> Artificial Sequence

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Yamamoto, Rika

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Yamamoto, Rika

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TARGET PROTEIN BY USING THE SAME

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<151> 2000-03-29

<150> JP 1999-288677
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